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PATENT
Attorney Docket No.: JHU1410-1

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicants:	Lee and McPherron	Art Unit:	1637
Application No.:	10/662,003	Examiner:	Y.J. Kim
Filed:	September 11, 2003	Conf. No.:	8520
Title:	METHODS FOR DETECTION OF MUTATIONS IN MYOSTATIN VARIANTS		

MAIL STOP AF
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

DECLARATION UNDER 37 C.F.R. § 1.131

Sir:

We, Se-Jin Lee and Alexandra McPherron, do hereby declare and state that:

1. We are co-inventors of the subject matter described and claimed in the U.S. Patent Application Serial No. 10/662,003, filed on September 11, 2003, entitled "Methods For Detection Of Mutations In Myostatin Variants," which is a continuation of U.S. Patent Application Serial No. 08/967,089, filed November 10, 1997, issued as U.S. Patent No. 6,673,534, which is a continuation-in-part of U.S. Patent Application Serial No. 08/862,445, filed on May 23, 1997, now abandoned.

2. We are familiar with the prosecution history of U.S. Patent Application Serial No. 10/662,003.

CERTIFICATION UNDER 37 CFR §1.8	
I hereby certify that the documents referred to as enclosed herein are being deposited with the United States Postal Service as first class mail on this date, **January 16, 2007,** in an envelope addressed to: Mail Stop AF, Commissioner for Patents, P. O. Box 1450, Alexandria, VA 22313-1450.	
Jason A. Gonzalez	January 16, 2007 *(Date)*

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3. We understand that the Examiner rejected claims 43-46 and 66-68 as being anticipated by and obvious over Grobet, et al., (U.S. Pat. No. 6,103,466, hereinafter "Grobet").

4. We have reviewed Grobet and are aware that it was filed on July 14, 1997, which is less than one year prior to November 10, 1997, the earliest priority date accorded to the above-identified patent application.

5. We are also aware that the Examiner has alleged that Grobet discloses a method of detecting the presence of a mutation in the myostatin gene, from a subject, wherein presence of the mutation is correlated with the subject having an increased muscle mass, wherein the mutation is a homozygous 11-base pair nucleotide deletion and the subject is Belgian Blue.

6. We respectfully submit that the claimed invention was conceived in the United States prior to July 14, 1997, the filing date of Grobet, as supported by the evidence which follows. All papers provided herewith are true copies of the original documents.

7. Exhibit 1 is a letter dated June 4, 1997, from Dr. Se-Jin Lee to Robert Curtis, President of MetaMorphix, Inc., enclosing drafts of two manuscripts. One of the manuscripts describes the Belgian Blue mutation and experimental methods for its detection. Exhibit 1 thus demonstrates that detection of the 11-bp deletion in the third exon of Belgian Blue myostatin, and correlation of the mutation to double-muscling of Belgian Blue, was conceived of and reduced to practice in the United States prior to July 14, 1997.

8. Exhibit 2 is a copy of a November 1997 publication by the undersigned inventors in *Proc. Natl. Acad. Sci., USA* (94:12457-12461 (1997)). As indicated on page 12457 of the publication, beneath the Title, the article was submitted for review for publication on August 12, 1997.

9. Exhibit 2 is the final version of the manuscripts of Exhibit 1. Accordingly, Exhibit 2 describes the deletion mutation in the myostatin gene of Belgian Blue cattle, as well as the point mutation in the myostatin gene of Piedmontese cattle that exhibit the double muscling phenotype (see Abstract). Exhibit 2 also discloses the amino acid sequences for myostatin proteins of several vertebrate species (see Figure 1, page 12458) and discloses that the nucleotide sequences encoding the myostatin proteins had been deposited in the GenBank database (see page 12457; footnote in right column). As such, Exhibit 2 demonstrates that, prior to July 14, 1997, the mutation in the myostatin gene of Belgian Blue cattle associated with double muscling had been identified by the inventors.

10. In summary, Exhibits 1 and 2 demonstrate that detection of the 11-bp deletion in the third exon of Belgian Blue myostatin, and correlation of the mutation to double-muscling of Belgian Blue was conceived of and reduced to practice in the United States prior to July 14, 1997.

11. The undersigned further declare that all statements made herein of knowledge are true and that all statements made on information and belief are believed to be true, and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine, or imprisonment, or both under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

12/11/06
Date

Se-Jin Lee

Date

Alexandra McPherron

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_______________ Date
_______________ Se-Jin Lee

12/14/06
Date

Alexandra McPherron
Alexandra McPherron

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June 25, 1997

Lisa A. Haile, Ph.D.
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Suite 1400
La Jolla, CA 92037

RE: Invention entitled ***Double-Muscling in the Belgian Blue Cattle Bred due to a Mutation in the Myostatin Gene*** JHU Ref.: DM-3272

Dear Lisa:

Nina asked that I forward the attached manuscript to you. Please prepare and file a U.S. patent application. Dr. Se-Jin Lee will be available if you have any questions.

Thank you.

Sincerely,

Cheryl

Cheryl D. Rexroad
Senior Technology Licensing Assistant
crexroad@welchlink.welch.jhu.edu

Enclosure

cc: Dr. Nina Ossanna (w/o enclosure)

3272

JOHNS HOPKINS
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JUN 4 1997

2AC

DATE ________________

TO:

NAME Robert Curtis

ADDRESS 2024 E. Monument St.

FAX # 5-1245

FROM:

NAME Se-Jin Lee

TELEPHONE # 4-0198

OF PAGES INCLUDING THIS PAGE ~~5~~ 15

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June 4, 1997

Robert Curtis
President, MetaMorphix, Inc.
c/o Office of Technology Licensing
Johns Hopkins University
2024 E. Monument St., Suite 2-100
Baltimore, MD 21205

Dear Bob,

Enclosed are drafts of two manuscripts that we are planning to submit soon. One of these describes the cloning of GDF-11. The other describes the Belgian Blue mutation. I will work with Lisa Haile to ensure that patent issues are secured.

Sincerely yours,

Se-Jin Lee, M.D., Ph.D.
Assistant Professor
Molecular Biology and Genetics

SJL:cam

enclosures

cc: L. Ellingsworth
W. Carlson w/o enclosures

DM 3272

Double-muscling in the Belgian Blue Cattle Breed Due to a Mutation in the Myostatin Gene

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There is a great clinical and agricultural interest in identifying genes that regulate muscle mass. We have recently identified myostatin (GDF-8), a new member of the transforming growth factor-ß superfamily of secreted growth and differentiation factors. Myostatin is expressed specifically in developing and adult skeletal muscle, and gene targeting studies in mice demonstrated an essential role for myostatin in the regulation of skeletal muscle mass. Myostatin null mice are viable and fertile but have over twice the normal mass of skeletal muscle due largely to an increased number of muscle fibers. Here we report the myostatin sequences of eight other vertebrate species and the identification of an 11 bp deletion in the coding sequence in the bovine myostatin gene in the Belgian Blue breed of cattle which are known to have an increase in muscle mass relative to conventional cattle. The similarity in phenotypes of the Belgian Blue cattle and myostatin null mice suggests that myostatin performs the same biological function in these two species and is a potentially useful target for genetic manipulation in other farm animals.

To clone the myostatin gene from other species, skeletal muscle cDNA libraries were screened with a mouse myostatin probe corresponding to the conserved carboxy-terminal region. An alignment of the predicted amino acid sequences of the murine, rat, human, baboon, porcine, ovine, bovine, chick and turkey genes (Fig. 1) shows that all of these proteins contain the expected signal sequence for secretion, proteolytic processing site of basic residues and conserved C-terminal cysteine residues found in all TGF-ß family members. Myostatin is highly conserved across species with the predicted myostatin

protein sequences being at least 9X% identical in the C-terminal reg:: 3 amino acids and at least 9X% identical overall when compared pairw:. : the C-terminal amino acid changes are conservative except in the bo sequence (cloned from the Holstein breed) where there are 2 non-co amino acid changes, K to E and E to G. It is not known what, if any, e changes may have on protein folding or activity.

To see if myostatin is a candidate gene for any disease or trait. human myostatin gene was mapped to chromosome 2 by amplificatic region myostatin sequences by PCR on a NIGMS mapping panel of rodent/human hybrids and detection with an oligo specific for human (Fig. 2a). This localization was confirmed by FISH to chromosome 2 2b). A mutation in the Belgian Blue cattle breed, designated muscula hypertrophy (*mh*), maps to bovine chromosome 2 (Charlier *et al.*, 199 region of human chromosome 2 is syntenic with bovine chromosome (Solinas-Toldo *et al.*, 1995). The *mh* mutation causes an average inc muscle mass of 20%, a decrease in intramuscular fat and connective a decrease in mass of most other organs (Hanset, 1991). Like the m null mice, the increase in muscle mass may actually be due to hyper rather than hypertrophy (). The *mh* locus segregates as a single aut recessive locus although there appears to be some increase in musc heterozygotes (). Since the *mh* locus maps near bovine type III colla (COL3A1) (Fisher *et al.*, 1997) and human COL3A1 maps to human chromosome 2q31-q32.3 (Povey and Falk, 1989) near human myost myostatin likely maps near the *mh* locus.

The similarities in phenotype between the myostatin null mice Belgian Blue cattle breed and the similar map positions of the myost and the *mh* locus suggested the bovine homolog of myostatin as a ca

gene for the *mh* mutation. To see if bovine myostatin is mutated in the Belgian Blue breed, all three exons of the gene from a Belgian Blue bull were amplified by PCR, subcloned and sequenced. The Belgian Blue myostatin coding sequences are identical to the Hostein sequences except for a deletion of nucleotides 937-947 in the third exon which encodes the mature C-terminal region. This 11 nucleotide deletion results in a frame-shift that ends with a stop codon 13 amino acids downstream. The deletion would be expected to be a null mutation since it occurs after only the first 7 amino acids of the C-terminal region resulting in a loss of 102 amino acids. This mutation is similar to the targeted mutation in myostatin null mice in which the exon carrying the C-terminal region was deleted (McPherron *et al.*, 1997).

Unlike in cattle, a myostatin null mutation in mice does not cause a reduction in sizes of internal organs, and the muscle mass in myostatin-deficient mice is increased at least 200% compared to 20% in the Belgian Blue breed. This suggests that cattle may be nearer to a maximal limit of muscle size after generations of selective breeding for large muscle mass, unlike mice, which have not been similarly selected. In this regard, we noticed earlier that even the Holstein sequence contains two amino acid differences in the C-terminal region compared to all other species examined. While the functional significance of these differences are unknown, it is possible that these two changes represent a partial loss-of-function allele.

For agriculture, there are some disadvantages in the Belgian Blue breed, namely the need for cesarean section and tendency for respiratory infections (Hanset, 1991). However, the reduction in food consumption and larger muscle mass offset these drawbacks (). The fact that a severe mutation in the myostatin gene in cattle results in animals that are still viable, fertile and produce high-quality meat demonstrates the feasibility of producing an increase in muscle

mass in other meat animals such as in the sheep, pig, chicken and turkey b disrupting the myostatin gene. The high degree of sequence conservation animals ranging from mammals to birds to fish suggests that the biological function of myostatin has been conserved widely in the animal kingdom as

MATERIALS AND METHODS

Cloning of myostatin. RNA isolation, twice polyA+ selection, cDNA libra construction and screening and analysis of cDNA clones were carried out a described (McPherron and Lee, 1993). cDNA libraries were screened with murine myostatin probe corresponding to the C-terminal 109 amino acids (McPherron *et al.*, 1997). Bovine (Holstein) and human genomic libraries w made in lambda Zap (Stratagene) according to the manufacturers instruc and screened ....

Northern analysis. Twenty µg of total RNA were elecrophoresed, blotte and hybridized as described (McPherron and Lee, 1993).

Mapping. PCR was performed on 0.5 µg of DNA from each cell type from NIGMS mapping panel using primers 5'-CGCGGATCCGTGGATCTAAATGAGAACAGTGAGC-3' and 84ACM 5'-CGCGAATTCTCAGGTAATGATTGTTTCCGTTGTAGCG-3' at 94°C for 1 m 60°C for 2 min. and 72°C for 2 min. for 40 cycles. PCR products were electrophoresed, blotted to nitrocellulose, hybridized with kinased oligo 5'-ACACTAAATCTTCAAGAATA-3' in 1XDenhardt's, 0.05% sodium pyrophosphate, 6XSSC and 100 µg ml^{-1} yeast tRNA at 42°C and washed 6XSSC, 0.05% sodium pyrophosphate at 50°C. FISH was performed on human metaphase spreads (BIOS, New Haven, CT) using a X kb digoxyge labeled human myostatin probe.

Sequencing of Belgian Blue DNA. Blood from cattle was spun at 5,000 rpm for 15 min., resuspended in 150 mM NaCl, 100 mM EDTA, digested with 1 mg ml^{-1} proteinase K and 1% SDS at 44°C and purified on a CsCl gradient. Exons were amplified by PCR from 1 µg genomic DNA using primer pairs 133ACM 5'-CGCGGATCCTTTGGCTTGGCGTTGCTCAAAAGC-3' and 134ACM 5'-CGCGGATCCTTCTCATGAACACTAGAACAGCAG-3' (exon 1), 135ACM 5'-CGCGGATTCGATTGATATGGAGGTCGTTCG-3' and 136ACM 5'-CGCGGATCCGGAAACTGGTAGTTATTTTTCACT-3' (exon 2) and 137ACM 5'-CGCGGATCCGAGGTAGGAGAGTGTTTTGGGATC-3' and 138ACM 5'-CGCGGATCCCACAGTTTCAAAATTGTTGAGGGG-3' (exon 3) at 94°C for 1 min., 50°C for 2 min. and 72°C for 2 min. for 40 cycles. PCR products were digested with BamHI, subcloned in pBS and sequenced.

REFERENCES

Fisher, S.R.

Povey, S.

Charlier, C.

Salinas-Toldo

McPherron, A.C. & Lee, S-J. "GDF-3 and GDF-9: two new members of the transforming growth factor-ß superfamily containing a novel pattern of cysteines" J. Biol. Chem. 268, 3444-3449 (1993).

McPherron, A.C., Lawler, A.M. & Lee, S-J. "Regulation of skeletal muscle mass in mice by a new member of the TGF-ß superfamily" Nat. 387, 8-16 (1997).

Hanset, R.

Proc. Natl. Acad. Sci. USA
Vol. 94, pp. 12457–12461, November 1997
Genetics

Double muscling in cattle due to mutations in the myostatin gene

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Communicated by Daniel Nathans, Johns Hopkins University School of Medicine, Baltimore, MD, August 26, 1997 (received for review August 12, 1997)

ABSTRACT **Myostatin (GDF-8) is a member of the transforming growth factor β superfamily of secreted growth and differentiation factors that is essential for proper regulation of skeletal muscle mass in mice. Here we report the myostatin sequences of nine other vertebrate species and the identification of mutations in the coding sequence of bovine myostatin in two breeds of double-muscled cattle, Belgian Blue and Piedmontese, which are known to have an increase in muscle mass relative to conventional cattle. The Belgian Blue myostatin sequence contains an 11-nucleotide deletion in the third exon which causes a frameshift that eliminates virtually all of the mature, active region of the molecule. The Piedmontese myostatin sequence contains a missense mutation in exon 3, resulting in a substitution of tyrosine for an invariant cysteine in the mature region of the protein. The similarity in phenotypes of double-muscled cattle and myostatin null mice suggests that myostatin performs the same biological function in these two species and is a potentially useful target for genetic manipulation in other farm animals.**

The transforming growth factor β superfamily encompasses a large group of secreted growth and differentiation factors that play important roles in regulating development and tissue homeostasis (1). We have recently described a member of this family, myostatin, that is expressed specifically in developing and adult skeletal muscle and functions as a negative regulator of skeletal muscle mass in mice (2). Myostatin null mice generated by gene targeting show a dramatic and widespread increase in skeletal muscle mass. Individual muscles in myostatin null mice weigh 2- to 3-fold more than those of wild-type mice, primarily due to an increased number of muscle fibers without a corresponding increase in the amount of fat. To pursue potential therapeutic and agricultural applications of increasing muscle mass by inhibition of myostatin activity, we have been characterizing myostatin in animals other than mice. Here we report that the myostatin gene is highly conserved among vertebrate species and that two breeds of cattle that are characterized by increased muscle mass (double muscling), Belgian Blue (3) and Piedmontese (4), have mutations in the myostatin coding sequence. These results demonstrate that the function of myostatin has been highly conserved among vertebrates.

METHODS

Cloning of Myostatin. Poly(A)-containing RNA was isolated from human (obtained from the International Institute for the Advancement of Medicine, Exton, PA), Holstein cow, sheep (Ruppersberger and Sons, Baltimore), pig (Bullock's Country Meats, Westminster, MD), White Leghorn chicken (Truslow Farms, Chestertown, MD), turkey (kindly provided by D. Boyer and D. Miller, Wampler Foods, Oxford, PA) and zebrafish (kindly provided by S. Fisher and M. Halpern, Carnegie Institution of Washington) skeletal muscle tissue as described (5). cDNA libraries were constructed in the λZAP II vector (Stratagene) according to the instructions provided by the manufacturer and screened without amplification. Rat and baboon skeletal muscle cDNA libraries and a bovine (Holstein) genomic library were purchased from Stratagene. Library screening and analysis of clones were carried out as described (5), except that the final washes were carried out in 25 mM sodium phosphate (pH 8.5), 0.5 M NaCl, 2 mM EDTA, and 0.5% SDS at 65°C.

Mapping. Fluorescence *in situ* hybridization was performed on human metaphase spreads (Bios, New Haven, CT) using a digoxigenin-labeled human genomic myostatin probe.

Sequencing of Bovine Genomic DNA. Blood from cattle was spun at 3,400 $\times g$ for 15 min, resuspended in 150 mM NaCl and 100 mM EDTA, and digested with 200 $\mu g \cdot ml^{-1}$ proteinase K and 1% SDS at 44°C. Semen (Select Sires, Rocky Mount, VA) was digested in 50 mM Tris (pH 8.0), 20 mM EDTA, 1% sarcosyl, 0.2 M 2-mercaptoethanol, and 200 $\mu g \cdot ml^{-1}$ proteinase K. DNAs were purified on a CsCl gradient. Exons were amplified by PCR from 1 μg genomic DNA using primer pairs 133ACM 5′-CGCGGATCCTTTGGCTTGGCGTTGCTCAAAAGC-3′ and 134ACM 5′-CGCGGATCCTTCTCATGAACACTAGAACAGCAG-3′ (exon 1), 135ACM 5′-CGCGGATCCGATTGATATGGAGGTGTTCGTTCG-3′ and 136ACM 5′-CGCGGATCCGGAAACTGGTAGTTATTTTTCACT-3′ (exon 2), and 137ACM 5′-CGCGGATCCGAGGTAGGAGAGTGTTTTGGGATC-3′ and 138ACM 5′-CGCGGATCCCACAGTTTCAAAATTGTTGAGGGG-3′ (exon 3) at 94°C for 1 min, 52°C for 2 min, and 72°C for 2 min for 40 cycles. PCR products were digested with *Bam*HI, subcloned into pBluescript, and sequenced.

Southern Blot Analysis of Mutant Sequences. One-fifth of exon 3 amplification products were electrophoresed on 2% agarose gels, blotted to nylon membranes, hybridized with ^{32}P-labeled 13-mers as described (6), and washed in 30 mM sodium citrate, 300 mM NaCl, and 0.1% SDS. Primers used were 146 ACM 5′-ATGAACACTCCAC-3′ (Holstein wild-type sequence, nucleotides 936–948), 145ACM 5′-TTGTGACAGAATC-3′ (Belgian Blue mutation, nucleotides 931–936 with 948–954), 673SJL 5′-GAGAATGTGAATT-3′ (Holstein wild-type sequence, nucleotides 1050–1062), and 674SJL 5′-GAGAATATGAATT-3′ (Piedmontese mutation, G1056A).

RESULTS AND DISCUSSION

To clone the myostatin gene from other species, cDNA libraries were constructed from RNA isolated from skeletal

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Data deposition: The sequences reported in this paper have been deposited in the GenBank database [baboon (accession no. AF019619), bovine (accession no. AF019620), chicken (accession no. AF019621), ovine (accession no. AF019622), porcine (accession no. AF019623), rat (accession no. AF019624), turkey (accession no. AF019625), zebrafish (accession no. AF019626), and human (accession no. AF019627)].

A commentary on this article begins on page 12249.

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muscle tissue and screened with a mouse myostatin probe corresponding to the conserved C-terminal region, which is mature, active portion of the molecule. An alignment of the predicted amino acid sequences of murine, rat, human, baboon, bovine, porcine, ovine, chicken, turkey, and zebrafish myostatin, deduced from nucleotide sequence analysis of full-length cDNA clones, is shown in Fig. 1. All of these sequences contain a putative signal sequence for secretion and a putative RXXR proteolytic processing site (amino acids 263–266) followed by a region containing the conserved C-terminal cysteine residues found in all transforming growth factor β family members (1). As seen from this alignment, myostatin is highly conserved across species. In fact, the sequences of murine, rat, human, porcine, chicken, and turkey myostatin are 100% identical in the C-terminal region following the putative proteolytic processing site, and baboon, bovine, and ovine myostatin contain only one to three amino acid differences in the mature protein. Zebrafish myostatin is considerably more diverged and is only 88% identical to the others in this region.

The high degree of sequence conservation of myostatin across species suggests that the function of myostatin has also been conserved. To determine whether myostatin plays a role in regulating muscle mass in animals other than mice, we investigated the possibility that mutations in the myostatin gene might account for the increased muscle mass observed in double-muscled livestock breeds. Double muscling, which has been observed in many breeds of cattle for the past 190 years, appears to be inherited as a single major autosomal locus with several modifiers of phenotypic expression, resulting in incomplete penetrance (7). In the most extensively studied double-muscled breed of cattle, Belgian Blue, the double muscling phenotype (Fig. 2) segregates as a single genetic locus designated muscular hypertrophy (*mh*) (8). The *mh* mutation, which is partially recessive, causes an average increase in muscle mass of 20–25%, a decrease in mass of most other organs (9, 10), and a decrease in intramuscular fat and connective tissue (11). The *mh* locus is tightly linked to markers on a region of bovine chromosome 2 (12) that is syntenic to a region of human chromosome 2 (2q32) (13) to which we had mapped the human myostatin gene by fluorescence *in situ* hybridization (data not shown).

The similarities in phenotype between the myostatin null mice and the Belgian Blue cattle breed and the similar map positions of the myostatin gene and the *mh* locus suggested the bovine homolog of myostatin as a candidate gene for the *mh* locus. To determine whether the bovine myostatin gene is mutated in the Belgian Blue breed, all three exons of the gene from the full-blood Belgian Blue bull shown in Fig. 2 were amplified by PCR, subcloned, and sequenced. The Belgian Blue myostatin coding sequence was identical to the Holstein sequence except for a deletion of nucleotides 937–947 in the third exon (Fig. 3). This 11-nucleotide deletion causes a frame-shift which is predicted to result in a truncated protein that terminates 14 codons downstream of the site of the mutation. The deletion is expected to be a null mutation because it occurs after only the first 7 amino acids of the C-terminal region, resulting in a loss of 102 amino acids (amino acids 274–375). This mutation is similar to the targeted mutation in myostatin null mice in which the entire region encoding the mature protein was deleted (2). By Southern blot analysis, using oligonucleotides corresponding to the wild-type

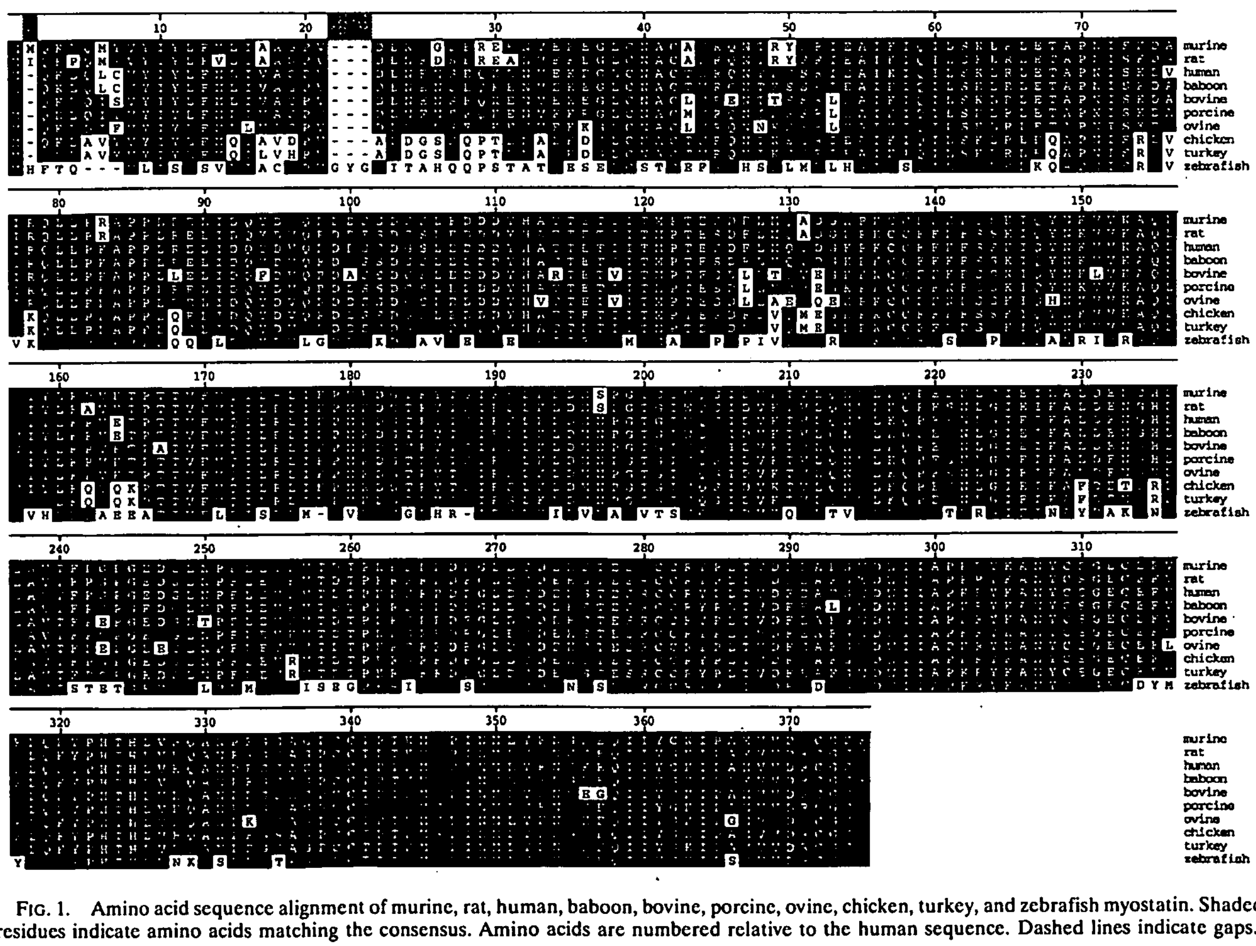

FIG. 1. Amino acid sequence alignment of murine, rat, human, baboon, bovine, porcine, ovine, chicken, turkey, and zebrafish myostatin. Shaded residues indicate amino acids matching the consensus. Amino acids are numbered relative to the human sequence. Dashed lines indicate gaps.

FIG. 2. A fullblood Belgian Blue bull showing the double muscling phenotype.

or mutant sequence, this mutation was found in both alleles in 14/14 fullblood Belgian Blue cattle examined (data not shown).

We also sequenced the myostatin gene in another cattle breed, Piedmontese, in which double muscling occurs at an extremely high frequency (4). The Piedmontese sequence contained 2 nucleotide changes relative to the Holstein sequence. One was a C to A transversion in exon 1, resulting in a conservative substitution of leucine for phenylalanine (amino acid 94). The second was a G to A transition in exon 3, resulting in a cysteine to tyrosine substitution in the mature region of the protein (amino acid 313) (Fig. 3). By Southern blot analysis, this mutation was found in both alleles in 10/10 double-muscled Piedmontese cattle examined. This mutation is likely to result in a complete or almost complete loss of function, as this cysteine residue is invariant not only among all myostatin sequences but also among all known members of the transforming growth factor β superfamily (1). This cysteine residue is known to be one of the amino acids involved in forming the intramolecular cystine knot structure in members of this superfamily for which the three-dimensional structure is known (14–17). Furthermore, when the corresponding cysteine in activin A (cysteine-44) was mutated to alanine, the mutant protein had only 2% of wild-type receptor binding and biological activity (18).

The similar map positions of the myostatin gene and the *mh* locus and the identification of relatively severe mutations in the myostatin gene of two different double-muscled cattle breeds suggest that these mutations are responsible for the double muscling phenotype. To further support this hypothesis, we analyzed DNA isolated from 120 individual fullblood or purebred cattle in 16 other breeds that are not classified as double-muscled (11 Angus, 11 Charolais, 10 Holstein, 10 Brown Swiss, 10 Polled Hereford, 10 Gelbvieh, 9 Simmental, 9 Jersey, 9 Guernsey, 9 Ayrshire, 7 Limousin, 4 Brahman, 4 Polled Shorthorn, 4 Red Angus, 2 Chianina, and 1 Texas

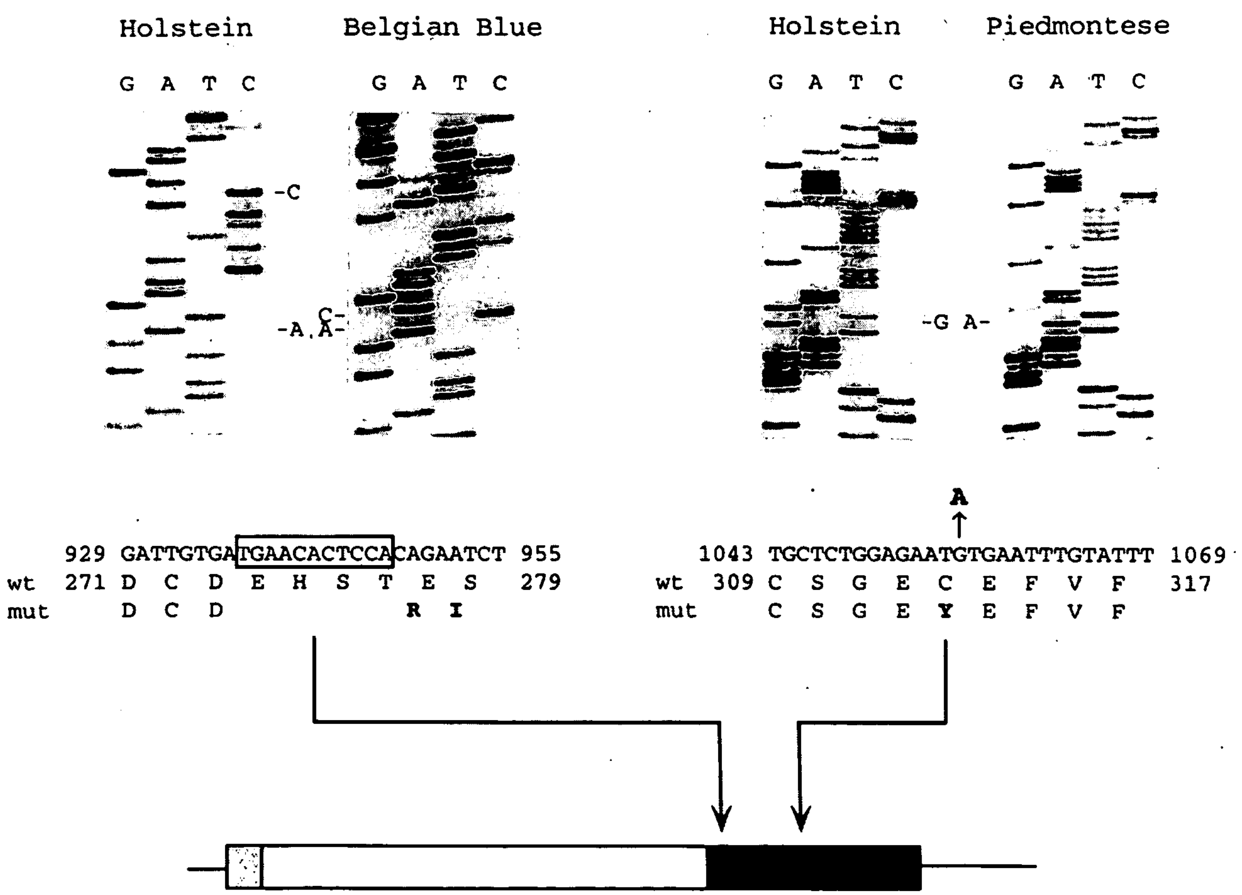

FIG. 3. Myostatin mutations in Belgian Blue (*Left*) and Piedmontese (*Right*) cattle compared with wild-type Holstein cattle. The nucleotides immediately preceding (A936) and following (C948) the Belgian Blue 11-nucleotide deletion are marked. Nucleotide and amino acid sequences are given below and numbered relative to wild type. The Belgian Blue 11-nucleotide deletion (Δ937–947) is boxed, and the Piedmontese G1056A transition is marked. Bold letters indicate nucleotide and amino acid changes. Arrows identify the locations of the mutations in the myostatin coding sequence. Shading indicates the signal sequence (gray), pro region (white) and mature C-terminal region (black).

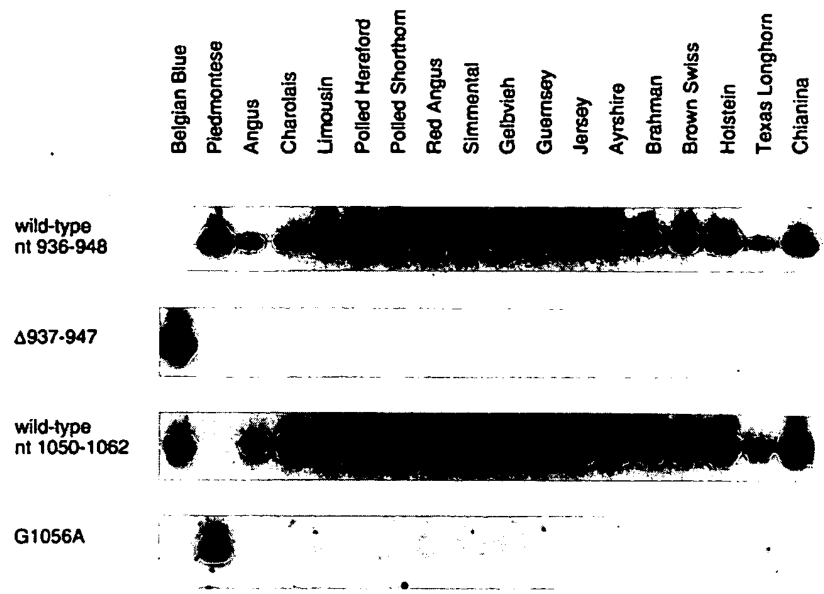

FIG. 4. Representative Southern blot hybridization showing the presence of the Belgian Blue and Piedmontese mutant sequences only in double-muscled breeds of cattle. Exon 3 PCR products were hybridized to oligonucleotide probes spanning the wild-type sequence of the region of the Belgian Blue mutation (top row), the Belgian Blue mutation Δ937–947 (second row), the wild-type sequence at nucleotide 1,056 (third row), and the Piedmontese mutant sequence at nucleotide 1,056 (bottom row). Differences in band intensity reflect differences in amounts of PCR products loaded, as judged by ethidium bromide staining (data not shown). Homozygosity for the mutations was seen only in double-muscled cattle and not in any conventional cattle as described in the text ($P < 0.001$ by χ^2).

Longhorn) for the presence of each of these mutations (Fig. 4). By Southern blot analysis, the cysteine to tyrosine substitution present in the Piedmontese breed was not detected in any of the 120 individuals. The 11-nucleotide deletion present in the Belgian Blue breed was detected in one allele of a single Red Angus non-double-muscled full-blood bull. In this regard, it has been suggested that the double muscling phenotype that is occasionally seen in many breeds may be due to a single mutation or very few mutations that migrated into many of the European breeds of cattle during the development of the modern breeds (7). Our results demonstrate that myostatin mutations which cause double muscling have occurred at least twice in cattle.

Finally, to rule out the presence of other myostatin mutations in non-double-muscled breeds, we determined the complete sequence of the myostatin coding region of 11 of these breeds (Angus, Charolais, Brown Swiss, Polled Hereford, Gelbvieh, Guernsey, Ayrshire, Limousin, Brahman, Polled Shorthorn, and Texas Longhorn). This analysis revealed only polymorphisms that were either silent changes in the coding sequences or were present in the introns and untranslated regions.

Unlike in mice, a myostatin null mutation in cattle causes a reduction in sizes of internal organs and only a modest increase in muscle mass (20–25% in the Belgian Blue breed as compared with 200–300% in myostatin-deficient mice). It is possible that cattle may be nearer to a maximal limit of muscle size after generations of selective breeding for large muscle mass, unlike mice, which have not been similarly selected. In this regard, even in cattle breeds that are not heavily muscled, the myostatin sequence contains two adjacent nonconservative amino acid differences (EG vs. KE) in the C-terminal region, compared with all other species examined. Although the functional significance of these differences is unknown, it is possible that these two changes represent a partial loss-of-function allele that became fixed in the population during many years of cattle breeding.

For agricultural applications, there are some disadvantages to double-muscled cattle, namely the reduction in female fertility, lower viability of offspring, and delay in sexual maturation (19). However, in the Belgian Blue breed, the increased muscle mass and increased feed efficiency largely offset these drawbacks (20). The fact that a null mutation in the myostatin gene in cattle results in animals that are still viable and fertile and produce high-quality meat demonstrates the potential value of producing an increase in muscle mass in other meat animals such as sheep, pig, chicken, turkey, and fish by disrupting myostatin function. Indeed, the high degree of sequence conservation in animals ranging from mammals to birds to fish suggests that the biological function of myostatin has been conserved widely throughout the animal kingdom.

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